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Hansenula polymorpha Swi1p and Snf2p are essential for methanol utilisation

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Abstract

We have cloned the *Hansenula polymorpha SWI1* and *SNF2* genes by functional complementation of mutants that are defective in methanol utilisation. These genes encode proteins similar to *Saccharomyces cerevisiae* Swi1p and Snf2p, which are subunits of the SWI/SNF complex. This complex belongs to the family of nucleosome-remodeling complexes that play a role in transcriptional control of gene expression.

Analysis of the phenotypes of constructed *H. polymorpha SWI1* and *SNF2* disruption strains indicated that these genes are not necessary for growth of cells on glucose, sucrose, or various organic nitrogen sources which involve the activity of peroxisomal oxidases. Both disruption strains showed a moderate growth defect on glycerol and ethanol, but were fully blocked in methanol utilisation. In methanol-induced cells of both disruption strains, two peroxisomal enzymes involved in methanol metabolism, alcohol oxidase and dihydroxyacetone synthase, were hardly detectable, whereas in wild-type cells these proteins were present at very high levels. We show that the reduction in alcohol oxidase protein levels in *H. polymorpha SWI1* and *SNF2* disruption strains is due to strongly reduced expression of the alcohol oxidase gene. The level of Pex5p, the receptor involved in import of alcohol oxidase and dihydroxyacetone synthase into peroxisomes, was also reduced in both disruption strains compared to that in wild-type cells.

Keywords: *Hansenula polymorpha*; Peroxisomes; Transcription regulation; SWI/SNF complex

1. Introduction

The methylotrophic yeast *Hansenula polymorpha* is able to grow on methanol as a sole carbon source. Growth on this compound is dependent on intact peroxisomes, which harbour key enzymes of methanol metabolism, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS) and catalase (CAT). Glucose-grown *H. polymorpha* cells generally contain one small peroxisome [1] with an unknown metabolic function. During growth on glucose, this organelle is redundant, because peroxisome-deficient *H. polymorpha* cells still can grow on this compound. Upon a shift of glucose-grown wild-type (WT) cells to fresh methanol media, these organelles serve as the initial target for import of newly induced AO, DHAS and CAT protein [1]. As a result the organelles grow and, when a certain size is reached, bud off new organelles that subsequently grow and divide. Therefore, methanol-grown *H. polymorpha* cells typically contain several, relatively large peroxisomes.

Abbreviations: AMO, amine oxidase protein; AO, alcohol oxidase protein; AOX, alcohol oxidase gene; CAT, catalase protein; DHAS, dihydroxyacetone synthase protein; GFP, green-fluorescent protein; Mut$^-$, methanol utilisation-deficient; PTS, peroxisomal targeting signal; ts, temperature-sensitive.

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Various attempts have been undertaken to mutationally dissect methanol metabolism in *H. polymorpha*. These studies have revealed that mutants unable to grow on methanol (Mut$^-$) mainly cluster in three groups, namely: (1) mutants defective in genes encoding enzymes of methanol metabolism (e.g., AO, DHAS, CAT, dihydroxyacetone kinase, enzymes of the xylulose-5-P cycle, etc.) [2–6]; (2) strains mutated in *PEX* genes that are essential for peroxisome biogenesis [7–13]; and (3) mutants affected in the transcriptional regulation of the genes mentioned under (1) and (2) [14]. Here, we describe the cloning of two novel *H. polymorpha* genes by functional complementation of two mutants, *77C* and *3P* that belong to the third group of Mut$^-$ mutants indicated above. These genes encode proteins that show homology to *Saccharomyces cerevisiae* Swi1p and Snf2p, proteins that are part of the SWI/SNF chromatin-remodeling complex. Using constructed *H. polymorpha* strains disrupted in *SWI1* or *SNF2*, we show that *H. polymorpha* Swi1p and Snf2p are important for the synthesis of two major peroxisomal enzymes involved in methanol metabolism, AO and DHAS. Our data also indicate that Swi1p and Snf2p influence the level of receptor protein Pex5p.

2. Materials and methods

2.1. Organisms and growth conditions

The *H. polymorpha* strains used in this study are listed in Table 1. *Escherichia coli* DH5α was used for cloning purposes and cultivated as described [15].

Solid media for growth of *H. polymorpha* cells contained either 1% yeast extract (Difco), 1% peptone (Difco) and 1% glucose (YPD) or 0.67% yeast nitrogen base without amino acids (Difco) supplemented with 1% glucose (YND) or 0.5% methanol (YNM). All these media contained 2% agar. For batch cultures, mineral medium [16] was used, supplemented with carbon source at concentrations shown in the text and 0.25% nitrogen source. For continuous cultures, mineral medium containing 0.25% glucose and 0.2% choline was used. Leucine was added when necessary to a final concentration of 20 mg/l.

All the strains were cultivated at 37 °C unless stated otherwise.

Table 1
Hansenula polymorpha strains used in this study

Strain	Reference
NCYC 495 (wild-type), *ura3 leu1.1*; *met6*	[17]
NCYC 495::P_{AOX} *GFP-SKL* (HF246) *leu1.1*	[6]
NCYC 495::P_{AMO} *GFP-SKL leu1.1*	(this study)
77C leu1.1	(this study)
3P leu1.1	[7]
snf2 (disruption with *URA3*) *leu1.1*	(this study)
swi1 (disruption with *URA3*) *leu1.1*	(this study)
snf2::P_{AMO} *eGFP-SKL*, *leu1.1*	(this study)
swi1::P_{AMO} *eGFP-SKL*, *leu1.1*	(this study)
aox (*aox-1*), *ade11*	[2]
dhas (disruption with pREMI-Z), *leu1.1*	[6]
cat (disruption with pREMI-Z), *leu1.1*	[6]
aox/*dhas*::P_{AMO} *eGFP-SKL*, *leu1.1*	(this study)
aox/*dhas*/*cat*, *leu1.1*	(this study)

2.2. Isolation of H. polymorpha 77C

Glucose-grown cells of *H. polymorpha* NCYC 495 strain HF246 (Table 1) were suspended in sterile water to density of 1×10^8 cells ml^{-1} and spread on solid YPD medium. Subsequently cells were irradiated using UV-light doses giving 1–10% survival. After 4–6 d incubation at 37 °C cells were screened for mutants defective in methanol-utilisation at 44 °C (ts Mut$^-$).

Genetic manipulations of *H. polymorpha* were performed as previously described [17].

2.3. Construction of strains

Strains NCYC 495 *aox*/*dhas* and *aox*/*dhas*/*cat* were obtained by crossing strains carrying single gene deletions/disruptions, namely *aox*, *dhas* and *cat* (Table 2). Diploids were subjected to random spore analysis; the resulting segregants were subjected to complementation analysis using *aox*, *dhas* and *cat* strains to determine the genotypes.

A *SWI1* disruption strain (*swi1*) was constructed by replacement of the gene between positions 373 and 1986 with the Hp*URA3* gene [18]. The disruption cassette was obtained as follows: two *SWI1* fragments were amplified by PCR (for primers see Table 2) containing suitable restriction sites. A fragment obtained upon digestion with *Sac*I and *Bgl*II was amplified using primers "Swi1 del 1" and "Swi1 del 2" (Table 2) and introduced downstream the Hp*URA3* gene in pBluescript-Hp*URA3*, digested with the same enzymes. The resulting plasmid was designated pPAK01. Subsequently a *SWI1* fragment, amplified using primers "Swi1 del 3" and "Swi1 del 4" (Table 2), was digested with *Xba*I–*Cla*I and ligated into pPAK01, upstream the Hp*URA3* gene. The 2.2-kb *Pst*I fragment of the resulting plasmid pPAK02 represented the *SWI1* disruption cassette and contained Hp*URA3* flanked by *SWI1* fragments of 0.5 kb.

The *SNF2* disruption strain (*snf2*) was constructed by replacing the region between positions 951 and 3573 with Hp*URA3*. The disruption cassette was constructed as follows: a *Sma*I–*Bgl*II fragment of the *SNF2* gene was introduced downstream Hp*URA3* in pBluescript-Hp*URA3* (*Not*I (Klenow fill-in)–*Bgl*II), resulting in pPAK03. Subsequently the *Sal*I–*Cla*I fragment of *SNF2* gene was ligated with pPAK03, digested with the same

Table 2
Oligonucleotide primers used in this study

Primer	Sequence 5′–3′	Restriction site
Swi1 del 1	TCCCCGCGGTGACCGCTCGTGCTGG	*Sac*II
Swi1 del 2	GAAGATCTGCGCAGTGTTGACGTTCG	*Bgl*II
Swi1 del 3	GCTCTAGACGTACTCACCAAGGTGC	*Xba*I
Swi1 del 4	CCATCGATCAAGGTGTTGCTACAGG	*Cla*I
KN1	CCCGGATCCATGGTGAGCAAGGGCGAG	*Bam*HI
KN2 [40]	CCCGTCGACTTACAGCTTCGACTTGTACAGCTCGTC	*Sal*I

enzymes. A 2.5-kb *Xho*I–*Mlu*I fragment of the resulting plasmid pPAK04 represented the *SNF2* disruption cassette containing Hp*URA3* flanked by a 5′ 0.4-kb and 3′ 0.3-kb fragment of *SNF2*.

Both disruption cassettes were transformed into *H. polymorpha* NCYC 495 *leu1.1 ura3*. Transformants were selected for uracil prototrophy and screened for the inability to grow on methanol at 37 °C. Proper integration of the disruption cassettes was confirmed by Southern-blot analysis.

The expression plasmid pFEM36, containing the *eGFP.SKL* reporter gene under control of the *H. polymorpha* amine oxidase promoter (P_{AMO}), was constructed as follows. By PCR, using primers KN1 and KN2 (Table 2), a *Bam*HI site was introduced upstream the *eGFP* start codon and an the sequence encoding SKL was introduced at the extreme C-terminus of eGFP followed by a *Sal*I site. The 0.8-kb PCR product was digested with *Bam*HI and *Sal*I and inserted into the same sites downstream the P_{AMO} in pHIPX5 [19]. The resulting plasmid was linearized with *Bsi* WI and transformed to *H. polymorpha* WT NCYC 495 *leu1.1*, *aox/dhas leu1.1*, *swi1 leu1.1* and *snf2 leu1.1*. Correct integration was confirmed by Southern-blot analysis.

To determine the activity of *AOX* promoter, WT NCYC 495 *leu1.1* strain as well as *swi1 leu1.1* and *snf2 leu1.1* mutant strains were transformed with plasmid pHIPX4 carrying bacterial β-lactamase gene behind 1.2-kb promoter of *AOX* gene [20].

2.4. Microscopy

Cells were fixed and prepared for electron microscopy [20] and fluorescence microscopy [21] as described previously. Immunolabeling was performed on ultrathin sections of unicryl-embedded cells using specific antibodies against amine oxidase and catalase, and gold-conjugated goat-anti-rabbit antibodies [20].

2.5. Biochemical methods

Preparation of crude extracts of *H. polymorpha* cells [21], SDS–polyacrylamide gel electrophoresis [22] and Western-blot analysis [23] were performed as detailed before. Nitrocellulose blots were decorated with specific polyclonal antibodies against various *H. polymorpha* proteins.

β-Lactamase activity measurements were carried out as described [20], using nitrocefine (Calbiochem, La Jolla, USA) as a substrate.

3. Results

3.1. Cloning of SWI1 and SNF2 by functional complementation

The original *H. polymorpha* mutant*3P* was included in a collection of mutants described before that were impaired to grow on methanol as sole source of carbon and energy (Mut^- phenotype) [7]. *H. polymorpha 77C* is a mutant of a novel collection of temperature-sensitive (ts) mutants that has been isolated in our laboratory, which display a Mut^- phenotype at 44 °C, but not at 37 °C. The corresponding genes were cloned by functional complementation using a *H. polymorpha* genomic library. The genomic fragment carrying the activity that complemented the Mut^- phenotype of mutant *3P* contained a single open reading frame (ORF) of 4383 base pairs that coded for a protein of 1461 amino acids. The protein product of this ORF showed the highest similarity (52%) to *S. cerevisiae* Snf2p (also designated Swi2/Gam1/Ric1/Tye3) and was more than 70% identical in the core domain between amino acid residues 485–1089 (Fig. 1(a)). Hereafter, we refer to this protein as HpSnf2p. The nucleotide sequence of the Hp*SNF2* gene was deposited at GenBank and assigned Accession No. AY303939. The open reading frame present in the DNA fragment that complemented the phenotype of mutant 77C coded for a protein of 878 amino acids (2634 base pairs). A database search identified the only homologous protein (26% homology) namely *S. cerevisiae* Swi1p (also named Adr6/Gam3/Lpa1) (Fig. 1(b)). Both proteins contain so-called ARID domain (AT-rich interaction domain), which is found in many eukaryotic transcription factors [24]. Furthermore, *S. cerevisiae* Swi1p contains an N-terminal domain as well as few internal domains that are lacking in *H. polymorpha* protein. We designated the cloned gene HpSWI1 and

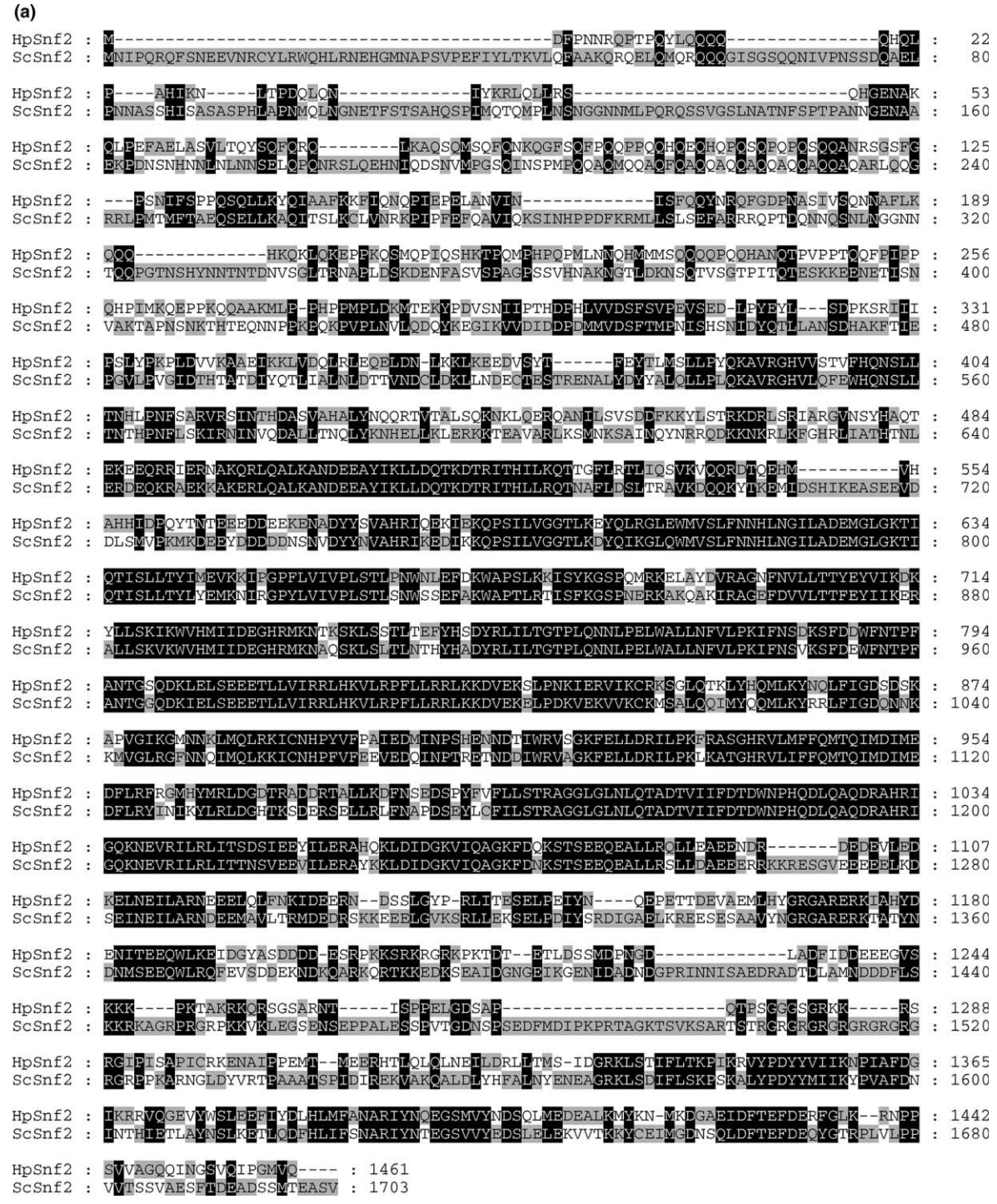


Fig. 1. (a) Alignment of *S. cerevisiae* Snf2 protein (ScSnf2) with its *H. polymorpha* homologue (HpSnf2, GenBank Accession No. AY303939). (b) Alignment of *S. cerevisiae* Swi1 protein (ScSwi1) with *H. polymorpha* homologue (HpSwi1, GenBank Accession No. AY303940). Alignments were performed using the Clustal X program [39] and viewed using GeneDoc program. Identical and similar residues are shown by black shading.

the translation product HpSwi1p. The nucleotide sequence was deposited at GenBank (Accession No. AY303940).

For the analysis of the role of *H. polymorpha* Snf2p and Swi1p homologues, *SWI1* and *SNF2* disruption strains (designated *swi1* and *snf2*) were constructed and crossed with the original mutants *77C* and *3P*, respectively. Both the resulting diploids and the segregants of these crossings invariably showed a Mut$^-$ phenotype, indicating that the *SWI1* and *SNF2* genes were indeed the mutated genes in *77C* and *3P* and do not represent suppressors.

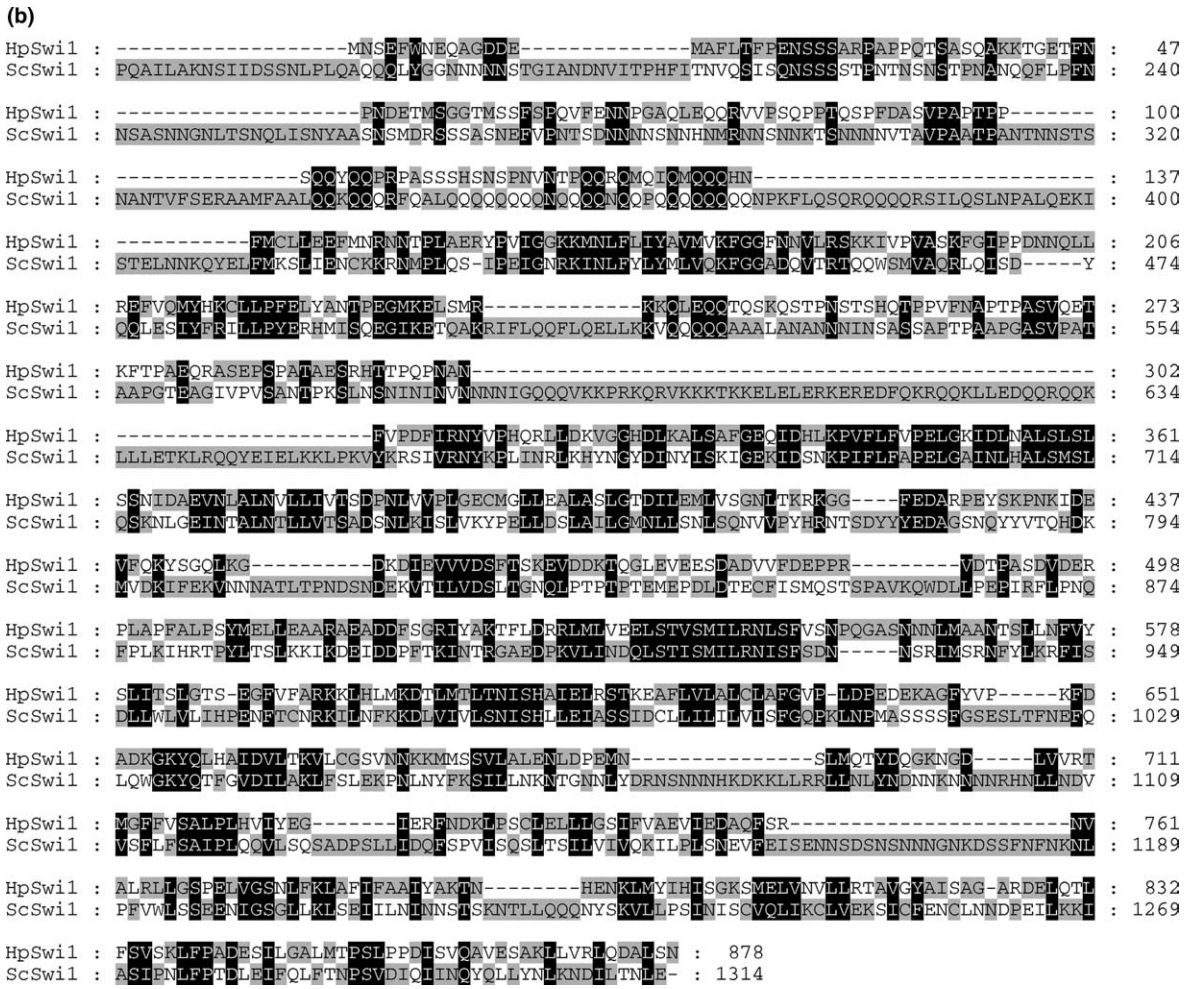


Fig. 1. (*continued*)

3.2. H. polymorpha snf2 *and* swi1 *display growth defects on methanol, glycerol and ethanol*

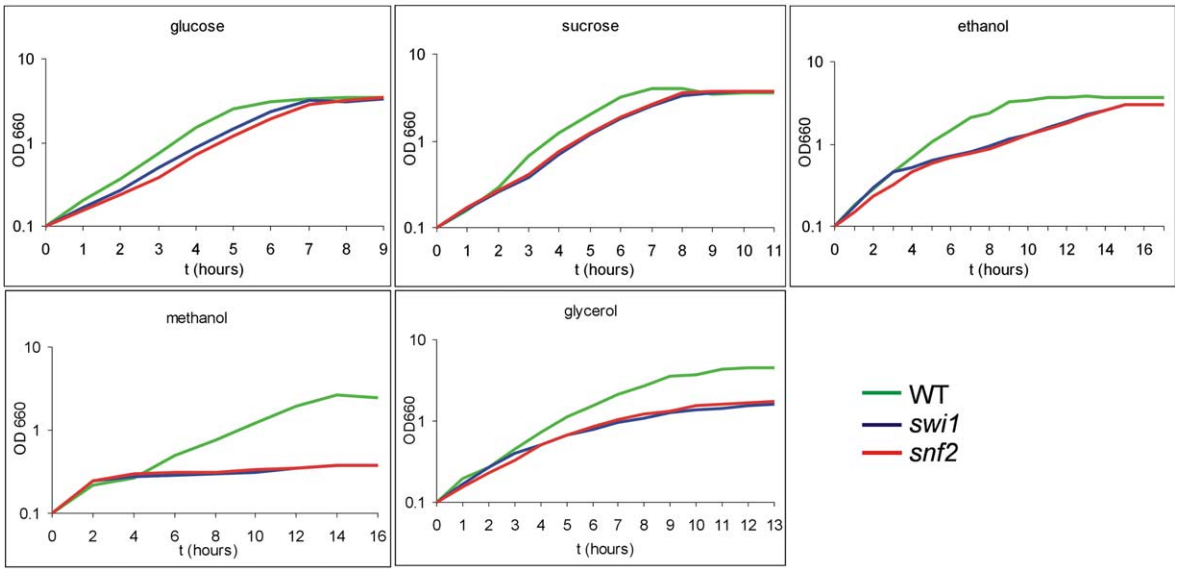
S. cerevisiae snf2 and *swi1* strains display growth defects on various carbon sources [25,26]. To elucidate, whether also the *H. polymorpha SWI1* and *SNF2* gene products play a role in other metabolic pathways, apart from methanol, growth of the *H. polymorpha swi1* and *snf2* cells on various media was analysed (Fig. 2).

Both mutant strains grew on glucose and sucrose at rates similar to WT and reached the same final optical density in the stationary growth phase, independent of the nitrogen source used (ammonium sulphate, Fig. 2; methylamine, D-alanine, data not shown). On ethanol and glycerol both *H. polymorpha* mutant strains showed some growth defect reflected in decreased growth rates in comparison with WT as well as lower final optical densities of the cultures. In the case of glycerol, we could not determine the doubling time of mutant strains, as the growth did not display a clear exponential phase. As expected, growth of *swi1* and *snf2* cells on methanol was fully impaired.

3.3. *Biochemical analysis of* swi1 *and* snf2 *cells*

In order to study the physiological basis of the Mut^- phenotype of *H. polymorpha swi1* and *snf2* mutants, samples of cells grown in glucose-limited chemostats in the presence of choline as nitrogen source were subjected to Western-blot analysis. Under these conditions derepression of peroxisomal enzymes of methanol metabolism as well as peroxisomal amine oxidase (AMO), a key enzyme of amine metabolism, is maximal [27]. Using antibodies against AO or DHAS, no specific bands were observed on Western blots prepared from crude extracts of both mutants strains, whereas strong bands were visible on blots prepared from identically grown WT cells (Fig. 3(a)). CAT and AMO protein levels were unchanged compared to that in WT controls. Analysis of peroxin levels showed minor (Pex14p, Pex3p) to rather strong (Pex5p) reductions in both mutants relative to WT controls (Fig. 3(b)). Pex5p is a soluble receptor protein, which is essential for sorting of proteins that contain a peroxisomal targeting signal 1 (PTS1) [28]. Since both AO and DHAS are PTS1 proteins and thus

(a)



(b)

	glucose		sucrose		ethanol		glycerol		methanol	
	t_d	OD_{final}	t_d	OD_{final}	t_d	OD_{final}	t_d	OD_{final}	t_d	OD_{final}
WT	0.96	3.27	0.95	3.62	1.8	3.68	1.8	4.54	3.31	2.46
swi1	1.27	3.27	1.22	3.69	4.37	3.06	n.d	3.56	∞	0.37
snf2	1.3	3.21	1.24	3.69	4.27	3.05	n.d	3.35	∞	0.38

Fig. 2. (a) Growth curves of *H. polymorpha swi1* and *snf2* and WT cells on different carbon sources. Cells of the three strains were pregrown in mineral medium with 0.25% glucose and shifted at the middle exponential growth phase to mineral medium supplemented with 0.3% carbon source (glucose, sucrose, ethanol, glycerol or methanol). (b) Doubling times (t_d) of *H. polymorpha* WT, *swi1* and *snf2* calculated from the growth curves presented in (a) and final growth yields (OD_{final}) in the stationary growth phase. Growth yield is expressed as optical density at 660 nm (OD_{660}) of cultures. N.d., not determined.

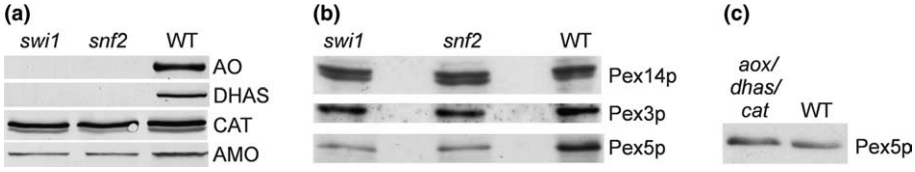


Fig. 3. Western-blot analysis of crude extracts prepared from WT, *swi1* and *snf2* cells grown in glucose-limited chemostats in the presence of choline as nitrogen source, using specific antisera against the indicated peroxisomal matrix proteins (a) and peroxins (b). (c) Levels of Pex5p in WT and the triple deletion mutant *aox*/*dhas*/*cat* grown in glucose-limited chemostats. Equal amounts of total protein extracts were loaded per lane.

targets for Pex5p, the question arose whether the reduction of Pex5p levels is a side effect caused by the strongly reduced PTS1-protein levels.

To address this question, we compared the Pex5p level in cells of a triple mutant unable to synthesize AO, CAT and DHAS (*aox*/*dhas*/*cat*) with that of identically grown WT cells. As shown in Fig. 3(c), Pex5p was not reduced in the triple mutant compared to WT. This demonstrates that a loss of the cargos AO and DHAS does not lead to a corresponding decrease in the Pex5p receptor.

3.4. *Methanol-induced* H. polymorpha swi1 *and* snf2 *cells show dramatic decrease in the activity of* AOX *promoter*

To analyse whether the observed reduction in AO levels in *swi1* and *snf2* strains is due to reduced expression levels, we studied the strength of the *AOX* promoter, by fusing it to a reporter gene encoding bacterial β-lactamase. Fig. 4(a) shows that in batch cultures grown under AO-inducing conditions, *snf2* cells show about 300-fold reduction in enzyme activity and in *swi1*

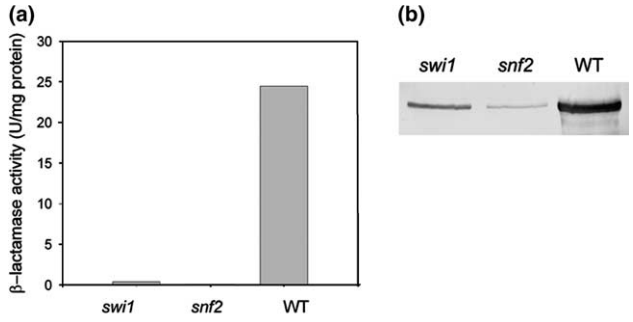


Fig. 4. (a) Activities of the *AOX* promoter in methanol-induced cells of *H. polymorpha* WT, *swi1* and *snf2*, determined by the level of enzyme activity of the P_{AOX}-driven bacterial β-lactamase. For enzyme induction, cells were grown for 15 h on mineral media containing 0.1% glycerol and 0.5% methanol. (b) Western-blot analysis of *H. polymorpha* WT, *swi1* and *snf2* cells grown as above. The blots were decorated using anti AO antibodies. Equal amounts of protein were loaded per lane.

cells, the β-lactamase activity is more than 50-fold lower than in WT cells. These findings indicate that the reduced levels of AO protein in *snf2*, *swi1* cells grown in batch (Fig. 4(b)) or in chemostat cultures (Fig. 3(a)) are due to reduced expression of the *AOX* gene.

3.5. *Peroxisome proliferation in* swi1 *and* snf2 *cells*

In peroxisome-deficient mutants of *H. polymorpha*, the metabolism of organic nitrogen sources that require the function of peroxisomal enzymes proceeds normally [29]. Hence, these metabolic pathways also efficiently function when these enzymes are mislocalized to the cytosol. Therefore, the fact that both *swi1* and *snf2* strains can utilise D-amino acids, primary amines and choline as sole nitrogen sources, does not imply that protein import to peroxisomes is not affected.

In order to determine whether disruption of the *SWI1* or the *SNF2* gene causes defects in peroxisome biogenesis, ultrathin sections of *swi1* and *snf2* cells were analysed by electron microscopy. As shown in Fig. 5, *snf2* cells contained peroxisomes, which were, however, reduced in diameter relative to WT organelles. This is the anticipated result because the most abundant peroxisomal proteins AO and DHAS are absent. Similar data were obtained for *swi1* cells (not shown). Immunocytochemistry performed on ultrathin sections of these cells using α-CAT and α-AMO antibodies revealed that specific labeling was confined to the peroxisomal profiles (Fig. 5(c), (d)). This indicates that both proteins, which are targeted by a PTS1 (CAT) or PTS2 (AMO) signal are normally sorted to peroxisomes and thus that PTS1 and PTS2 import is most likely not disturbed in *swi1* and *snf2* cells.

The proliferation of peroxisomes in both mutant strains was further analysed by fluorescence microscopy using the enhanced form of green-fluorescent protein (eGFP) [30] fused to the consensus PTS1 signal-SKL. The *eGFP-SKL* gene was placed under control of the

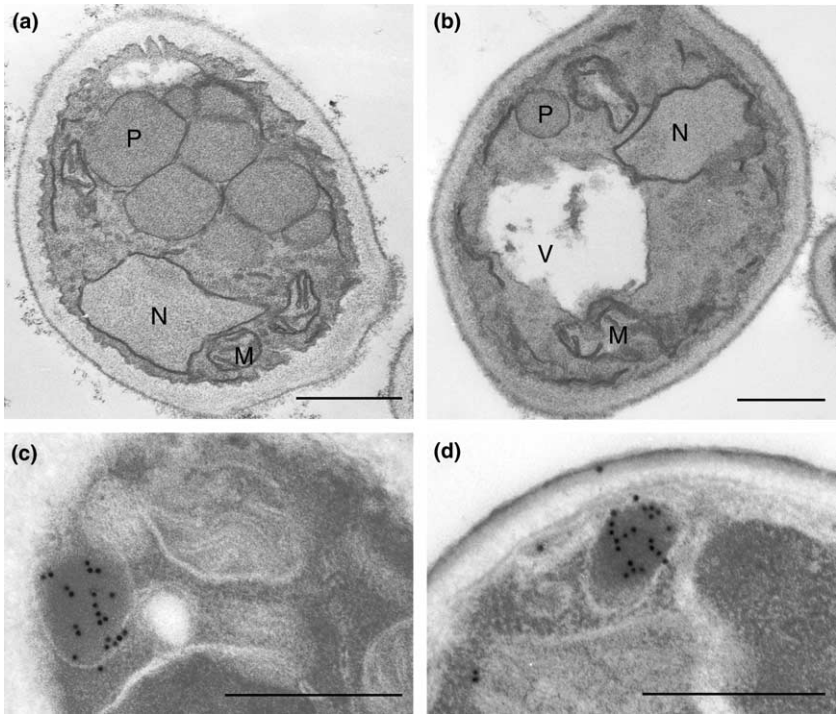


Fig. 5. Ultrastructural analysis of cells of the s*nf2* strain versus WT, grown in a chemostat on glucose/choline. Morphology of $KMnO_4$-fixed cells: wild- type (a), *snf2* (b). Immunolabeling of glutaraldehyde-fixed cells of *snf2* cells using α-amine oxidase (c) or α-catalase (d) antibodies. The bar represents 0.5 μm. M, mitochondrion; N, nucleus, P, peroxisome; V, vacuole.

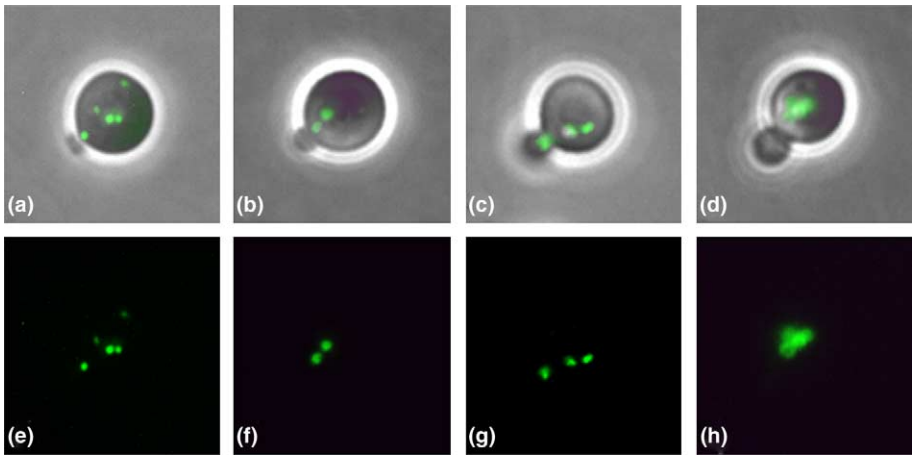


Fig. 6. Fluorescence microscopy of *swi1* (a, e), *snf2* (b, f), *aox/dhas* (c, g) and WT (d, h) cells producing the peroxisomal marker protein GFP.SKL. (a)–(d) Overlays of bright field and fluorescence images; (e)–(h) fluorescence images. Fluorescence of GFP.SKL in wild-type cells shows the typical pattern of clusters of peroxisomes. Cells were grown in mineral medium supplemented with 0.1% glycerol, 0.5% methanol and 0.25% methylamine.

amine oxidase promoter (P_{AMO} *eGFP-SKL*). Cells that were placed at conditions that induce peroxisome proliferation and synthesis of eGFP-SKL were analysed by fluorescence microscopy. As shown in Fig. 6, the number of organelles in both the *swi1* and *snf2* cells did not differ significantly from that in WT controls. In WT cells eGFP fluorescence is typically observed in clusters of peroxisomes. In *swi1*, *snf2* or *aox/dhas* cells peroxisomes were not clustered, but scattered over the cytosol.

4. Discussion

We have cloned two *H. polymorpha* genes by functional complementation of mutants defective in growth on methanol. The cloned genes encode proteins that are homologous to *S. cerevisiae* Swi1p and Snf2p, two components of the SWI/SNF complex, a large multisubunit complex, consisting of eleven components (reviewed in [31]). SWI/SNF complexes are found in all eukaryotes and involved in transcriptional regulation of specific genes. The complex functions in transcriptional activation by remodeling of nucleosomes, which results in access of transcription factors to their binding sites. In addition, SWI/SNF complexes play a direct role in transcriptional repression of certain genes by a distinct mechanism, which is not yet well understood [32].

Snf2p is one of the conserved core proteins of the SWI/SNF complex and contains DNA-dependent ATPase activity that triggers the onset of the remodeling reaction. Swi1p is not one of the core components. Whole-genome microarray studies revealed that *S. cerevisiae swi1* and *snf2* cells show similar expression profiles, in which the expression of the same genes is either up- or down regulated relative to WT [26]. These studies also revealed that in *S. cerevisiae* SWI/SNF modulates transcription of a small percentage (approx. 6%) of the genes [26,32].

In baker's yeast, *SNF2* was originally identified as a gene required for sucrose fermentation (sucrose nonfermenting [25]), whereas Swi1p (also designated Adr6p) was identified as being involved in expression of alcohol dehydrogenase isoenzyme 2 (Alcohol dehydrogenase regulation, [33]). Both *S. cerevisiae snf2* and *swi1* mutants grow slowly on various carbon sources as sucrose, galactose, lactate, glycerol or ethanol.

The phenotypes of *H. polymorpha swi1* and *snf2* are very similar to *S. cerevisiae swi1* and *snf2* with respect to growth on ethanol and glycerol. However, *H. polymorpha swi1* and *snf2* cells do not show a growth defect on sucrose. *H. polymorpha*, however, does not possess the *SUC2* gene (Gerd Gellissen, personal communication), which in baker's yeast is required for growth on sucrose and strongly down-regulated in *snf2* and *swi1* cells. Instead, *H. polymorpha* uses maltase [34], which apparently is not influenced by the absence of HpSwi1p and HpSnf2p. Therefore, normal growth of *H. polymorpha swi1* and *snf2* mutants on sucrose does not rule out the hypothesis that HpSwi1p and HpSnf2p are the true homologues of ScSwi1p and ScSnf2p. Instead, the similar phenotypes of *H. polymorpha swi1* and *snf2* and slow growth on ethanol and glycerol as well as the sequence homology to *S. cerevisiae* Snf2p and Swi1p proteins lend support to the view that also HpSwi1p and HpSnf2p are components of a SWI/SNF complex involved in regulation of multiple metabolic pathways.

Here, we demonstrated that the expression of the *AOX* gene was strongly reduced in the absence of HpSwi1p or HpSnf2p. As it has previously been shown by others [35], in glucose-repressed cells the *AOX* promoter is organised in nucleosomes, what prevents transcription. These nucleosomes must be removed upon methanol-induction, and this, according to our data,

involves Swi1p and Snf2p. The *DHAS* gene is known to be as tightly regulated as *AOX*. Although little is known about the mechanisms of this regulation, our data suggest that derepression of *DHAS* involves chromatin remodeling as well.

Our data also indicate that next to the two peroxisomal enzymes involved in methanol-metabolism, the level of Pex5p, which is essential for targeting of these proteins, may be regulated by the SWI/SNF complex.

Vallini et al. [14] and Genu et al. [36] recently also have identified several novel *H. polymorpha AOX* regulatory mutants. The phenotypes of these mutants, however, differ from *H. polymorpha swi1* and *snf2*. The mutants described by Vallini et al. [14] were specifically defective in methanol-utilisation except for mutant VM-13, which also showed a defect in growth on ethanol, glycerol and xylose and weak growth on methylamine. VM-13 differs from *H. polymorpha swi1* and *snf2*, which are not defective in utilisation of methylamine. Similar to *H. polymorpha swi1* and *snf2* cells, one of the mutants recently reported by Genu et al. [36] (Q1N-M8) also lacked detectable AO activity. Moreover, this strain was defective in growth on non-fermentable carbon sources, but growth on glucose was also reduced compared to WT. Further analysis has revealed that the growth defect on glucose was due to a block in mitochondrial respiration and that *AOX* transcription is repressed when respiration is blocked. Physiologically, this makes sense since energy generation during methylotrophic growth is fully dependent on mitochondrial respiration [37]. The observed down-regulation of *AOX* expression in *H. polymorpha snf2* and *swi1* can not be explained from defects in mitochondrial respiration, because both mutants show only a partial growth defect on ethanol and normally grow on glucose [36].

Our analysis of the levels of peroxisomal enzymes in *H. polymorpha snf2* and *swi1* strains revealed no decrease in CAT levels. That confirms earlier findings that *AOX* and *CAT* are not identically regulated although both promoters have the same UAS (at -480 in *AOX* and -410 in *CAT*). In the *AOX* promoter, this UAS is part of a nucleosome-free region [35] and therefore may not require chromatin remodeling for activator binding. Apparently the presumed SWI/SNF complex functions at another, nucleosome-containing region in the *AOX* promoter. Interestingly, it was recently reported that the sensitivity of the *AOX* TATA box (at -50) towards DNAse I is increased upon methanol induction [36]. Possibly, the SWI/SNF complex plays a role in this process.

The fluorescence studies indicated that deletion of *SWI1* or *SNF2* did not significantly affect peroxisome numbers (proliferation). The only difference observed concerned the size of the organelles that were reduced relative to WT peroxisomes. This was however, the expected result since two major peroxisomal proteins were absent.

A second remarkable feature of *swi1* and *snf2* cells was that peroxisomes were not clustered like in WT, but scattered throughout the cytosol. One reason could be that specific genes involved in peroxisome clustering are misregulated in the absence of Snf2p and Swi1p. Possible candidates are *S. cerevisiae PEX28* and *PEX29* that recently were shown to influence peroxisome-clustering [38]. Interestingly, peroxisomes were also not clustered in the double-deletion strain *aox/dhas*. The reason for this is not known.

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